Original Article

High Glucose Impairs Early and Late Endothelial Progenitor Cells by Modifying Nitric Oxide–Related but Not Oxidative Stress–Mediated Mechanisms

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OBJECTIVE—Endothelial progenitor cells (EPCs) are impaired in diabetes. This study aimed to investigate the direct effects of high glucose on EPCs.

RESEARCH DESIGN AND METHODS—Mononuclear cells isolated from healthy subjects were incubated with glucose/mannitol or drugs for EPC study. After 4 days of culture, attached early EPCs appeared. The monolayer late EPCs with cobblestone shape appeared at 2–4 weeks. Various immunofluroscence stainings were used to characterize the early and late EPCs. Senescence assay and the activity of endothelial nitric oxide synthase (eNOS) were determined. Migration and tube formation assay were done to evaluate the capacity for vasculogenesis in late EPCs.

RESULTS—Chronic incubation with high glucose but not mannitol (osmotic control) dose-dependently reduced the number and proliferation of early and late EPCs, respectively. High glucose enhanced EPC senescence and impaired the migration and tube formation of late EPCs. High glucose also decreased eNOS, FoxO1, and Akt phosphorylation and bioavailable nitric oxide (NO) in both EPCs. The effects of high glucose could be ameliorated by coincubation with NO donor sodium nitroprusside or p38 mitogen–activated protein kinase inhibitor and deteriorated by eNOS inhibitor or PI3K (phosphatidylinositol 3′-kinase) inhibitor. Antioxidants including vitamin C, *N*-acetylcysteine– and polyethylene glycol (PEG)-conjugated superoxide dismutase, and PEG-catalase had no effects, whereas pyrrolidine dithiocarbamate, diphenyleneiodonium, apocynin, and rotenone even deteriorated the downregulation of both EPCs.

CONCLUSIONS—High glucose impaired the proliferation and function of early and late EPCs. NO donor but not antioxidants reversed the impairments, suggesting the role of NO-related rather than oxidative stress–mediated mechanisms in hyperglycemia-caused EPC downregulation. ***Diabetes*** **56:1559–1568, 2007**

Hyperglycemia, associated with endothelial dysfunction and reduced new blood vessel growth, is a primary cause of vascular complications in diabetes (1,2). There is increasing evidence that neovascularization in adults is not solely the result of proliferation of local endothelial cells (angiogenesis) but also involves bone marrow–derived circulating endothelial progenitor cells (EPCs) in the process of vasculogenesis (3). EPCs can be isolated, cultured, and differentiated ex vivo from the circulating mononuclear cells (MNCs) and exhibit characteristic endothelial properties and markers (4,5). Currently, two types of EPCs, namely early and late EPCs, can be derived and identified from peripheral blood. Although they might have different roles in neovasculogenesis, most of the previous studies mainly focused on early rather than late EPCs (6,7). It was recently shown that the number and function of circulating EPCs could be reduced in patients with cardiovascular risk factors such as hyperglycemia, hypertension, or smoking (8). The reduced number and function of early EPCs were also found to associate with the pathogenesis of diabetes vascular complications (9) in either type 1 (10) or type 2 (11,12) diabetes.

Expression and phosphorylation of endothelial nitric oxide synthase (eNOS) are known to be essential for the survival, migration, and angiogenesis of either EPCs or endothelial cells (13,14). Nitric oxide (NO) derived from eNOS has been identified as promoting the mobilization of EPCs from the bone marrow through nitrosylation and elevated vascular endothelial growth factor (VEGF) expression (15). VEGF was shown to feed back on Akt and phosphorylation of eNOS at the serine residue 1,177 ($Ser^{1,177}$) and contribute to postnatal neovascularization by mobilizing bone marrow–derived EPCs (16) through eNOS-dependent effects (15). Thus, NO could be critical to the regulation of EPC functions. Recently, it was demonstrated that hyperglycemia-induced impairment of early EPCs could be restored via the modulation of p38 mitogen–activated protein kinase (MAPK) (17,18) and Akt/FoxO1

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acLDL, acetylated LDL; DiI-acLDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acLDL; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; L-NAME, L-N^{g}-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; MNC, mononuclear cell; PEG, polyethylene glycol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; UEA-1, ulex europaeus agglutinin.

signaling (19). However, the effects and mechanisms on late EPCs are not known.

Excessive generation of reactive oxygen species (ROS) and reactive nitrogen species may contribute to endothelial dysfunction and play a critical role in the progressive deterioration of vessel structure and function (20,21). While low levels of ROS are essential and participate in important intracellular signaling pathways (22), excessive generation of ROS may result in cytotoxic oxidative stress. In patients with diabetes, the production of ROS is increased, which could contribute to the onset and development of diabetes vascular complications (23). On the other hand, hyperglycemia may impair endothelial NO production (24,25) and alter the intracellular reduction-oxidation state in endothelial cells, which is believed to associate with endothelial dysfunction and vascular complications in diabetes (26). Recently, hyperglycemia was shown to reduce the number and function of circulating blood–derived progenitor cells, mainly early EPCs, both in vivo (10–12) and in vitro (17,27) However, it is also suggested that human EPCs, with high intrinsic expression of antioxidant enzymes (28) such as manganese superoxide dismutase, could tolerate oxidative stress (29). Thus, the influence of hyperglycemia on intrinsic NO and redox status in both early and late EPCs should be further clarified. This study was thus conducted to examine the direct effects of high glucose on the number/proliferation and functional activities of both early and late EPCs and, more importantly, to investigate the individual roles of NO- or oxidative stress–related mechanisms in high glucose–induced impairments, if there are any.

RESEARCH DESIGN AND METHODS

EPC isolation and cultivation. Total MNCs were isolated from 40 ml peripheral blood of healthy young human volunteers by density gradient centrifugation with Histopaq-1077 (density 1.077 g/ml; Sigma). MNCs (1×10^7) were plated in 2 ml endothelial growth medium (EGM-2 MV; Cambrex) (Fig. 1*A*), with supplements (hydrocortisone, R^3-insulin-like growth factor 1, human endothelial growth factor, VEGF, human fibroblast growth factor, gentamicin, amphotericin B, vitamin C, and 20% fetal bovine serum) on fibronectin-coated six-well plates at 37°C in a 5% CO_2 incubator. Under daily observation, after 4 days of culturing, medium were changed and nonadherent cells were removed; attached early EPCs appeared, elongated with a spindle shape (Fig. 1*B*). Thereafter, medium were replaced every 3 days, and each colony/cluster was followed-up. A certain number of early EPCs can continue to grow into colonies of late EPCs, which emerge 2–4 weeks after start of MNC culture. The late EPCs exhibited "cobblestone" morphology and monolayer growth pattern typical of mature endothelial cells at confluence (Fig. 1*C*) (6). Cells were incubated on the day of isolation with glucose/mannitol or drugs without changing the medium for early EPC study; cells under passage 3 were used for late EPC study.

EPC colony-forming assay. In another set of study, isolated MNCs were resuspended in growth medium (EndoCult; StemCell Technologies, Vancouver, Canada), and in total 5×10^6 MNCs were preplated in fibronectin-coated six-well plates in duplicate. After 2 days, the nonadherent cells were collected, and 1×10^6 cells were replated onto a fibronectin-coated 24-well plate. On day 5 of the assay, the number of colony-forming units per well was counted for each sample. A colony of EPCs was defined as a central core of round cells with elongated sprouting cells at the periphery. All colonies were counted manually in a minimum of three wells by two independent investigators under blind condition. Cells were incubated on the day of isolation with glucose/mannitol.

EPC characterization. The early EPCs were characterized as adherent cells double positive for acetylated LDL (acLDL) uptake and lectin binding by direct fluorescent staining (8). Briefly, the adherent cells were first incubated with 2.4 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acLDL (DiI-acLDL; Molecule Probe) for 1 h and then fixed in 2% paraformaldehyde and counterstained with 10 μg/ml fluorescein isothiocyanate–labeled lectin from ulex europaeus agglutinin (UEA-1) (Sigma). The late EPC-derived outgrowth endothelial cell population was also characterized by immunofluroscence staining for the expression of VE-cadherin, von Willebrand factor, PECAM-1 (platelet/endothelial cell adhesion molecule-1) (CD31), CD34, kinase insert domain receptor (KDR)/VEGF receptor 2, and AC133 (CD133) (Santa Cruz). The fluorescent images were recorded under a laser scanning confocal microscope.

EPC number and proliferation assay. The number of early EPCs and the proliferation of late EPCs were determined by direct counting six random high-power microscope fields (×100) and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively. Cells were cultured in plate with various concentrations of glucose or mannitol (as osmotic control). After being cultured for 4 days, the amounts of early EPCs were counted and late EPCs were supplemented with MTT (0.5 mg/ml, Sigma) and incubated for 4 h for proliferation assay. The blue formazen was dissolved with dimethyl sulfoxide and measured at 550/650 nm.

EPC senescence assay. The cellular aging was determined with a Senescent Cells Staining Kit (Sigma). Briefly, after washing with PBS, both early and late EPCs were fixed for 6 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and then incubated for 12 h at 37°C without CO_2 with fresh _X-gal staining solution (1 mg/ml X-gal, 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, and 2 mmol/l $MgCl_2$; pH 6). After staining, blue-stained cells and total cells were counted and the percentage of β-galactosidase–positive cells was calculated (30).

EPC migration assay. The migratory function of late EPCs that is essential for vasculogenesis was evaluated by a modified Boyden chamber (Transwell, Coster) assay (31). In brief, isolated EPCs were detached as described above with trypsin/EDTA, and then 4×10^4 EPCs were placed in the upper chamber of 24-well Transwell plates with polycarbonate membrane (8-μm pores) with serum-free endothelial growth medium; VEGF (50 ng/ml) in medium was placed in the lower chamber. After incubation for 24 h, the membrane was washed briefly with PBS and fixed with 4% paraformaldeyde. The upper side of membrane was wiped gently with a cotton ball. The membrane was then stained using hematoxylin solution and removed. The magnitude of migration of late EPCs was evaluated by counting the migrated cells in six random high-power (×100) microscope fields.

EPC tube formation assay. Tube formation assay was performed on late EPCs to assess the capacity for EPC vasculogenesis, which is believed to be important in new vessel formation. In vitro tube formation assay was performed with In Vitro Angiogenesis Assay Kit (Chemicon) (31). The protocol was according to the manufacturer's instructions. In brief, ECMatrix gel solution was thawed at 4°C overnight, then mixed with ECMatrix diluent buffer, and placed in a 96-well plate at 37°C for 1 h to allow the matrix solution to solidify. EPCs were harvested as described above with trypsin/EDTA, then 1×10^4 EPCs were placed on matrix solution with EGM-2 MV medium with glucose or mannitol, and incubated at 37°C for 16 h. Tubule formation was inspected under an inverted light microscope (×100). Four representative fields were taken and the average of the total area of complete tubes formed by cells was compared by computer software, Image-Pro Plus.

Western blot analysis. Protein extracts were prepared as previously described (32). Briefly, EPCs were lysed in a buffer (62.5 mmol/l Tris-HCl, 2% SDS, 10% glycerol, 0.5 mmol/l PMSF, 2 μg/ml aprotinin, pepstatin, and leupeptin). The protein lysates were subjected to SDS-PAGE, followed by electroblotting onto PVDF membrane. Membranes were probed with monoclonal antibodies that directed to phosphorylated eNOS (*p*-eNOS), eNOS, iNOS, α-tubulin (Chemicon), p-FoxO1, FoxO1, p-Akt, and Akt (Cell Signaling). Bands were visualized by chemiluminescence detection reagents (NEN). Densitometic analysis was conducted with ImageQuant (Promega) software.

Measurement of nitrite and intracellular cGMP content. After incubation of EPCs with glucose/mannitol for 4 h, the conditioned medium and cell lysates were measured for nitrite level and cGMP content by Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2% phosphoric acid] and cGMP EIA Kit (Cayman), respectively.

Statistical analysis. Data are presented as means ± SE. Intergroup comparisons were performed by Student's t test or one-way ANOVA. Probability values of $P < 0.05$ were considered statistically significant.

RESULTS

Characterization of EPCs. EPCs were originated from peripheral blood MNCs of healthy subjects as previously described (3). The peripheral blood MNCs that initially seeded on fibronectin-coated wells were round (Fig. 1*A*). After changing medium on day 4, attached early EPCs appeared and elongated with spindle shape (Fig. 1*B*). Late EPCs with cobblestone-like morphology similar to mature endothelial cells were grown to confluence (Fig. 1*C*). A colony of EPCs was defined as a central core of round

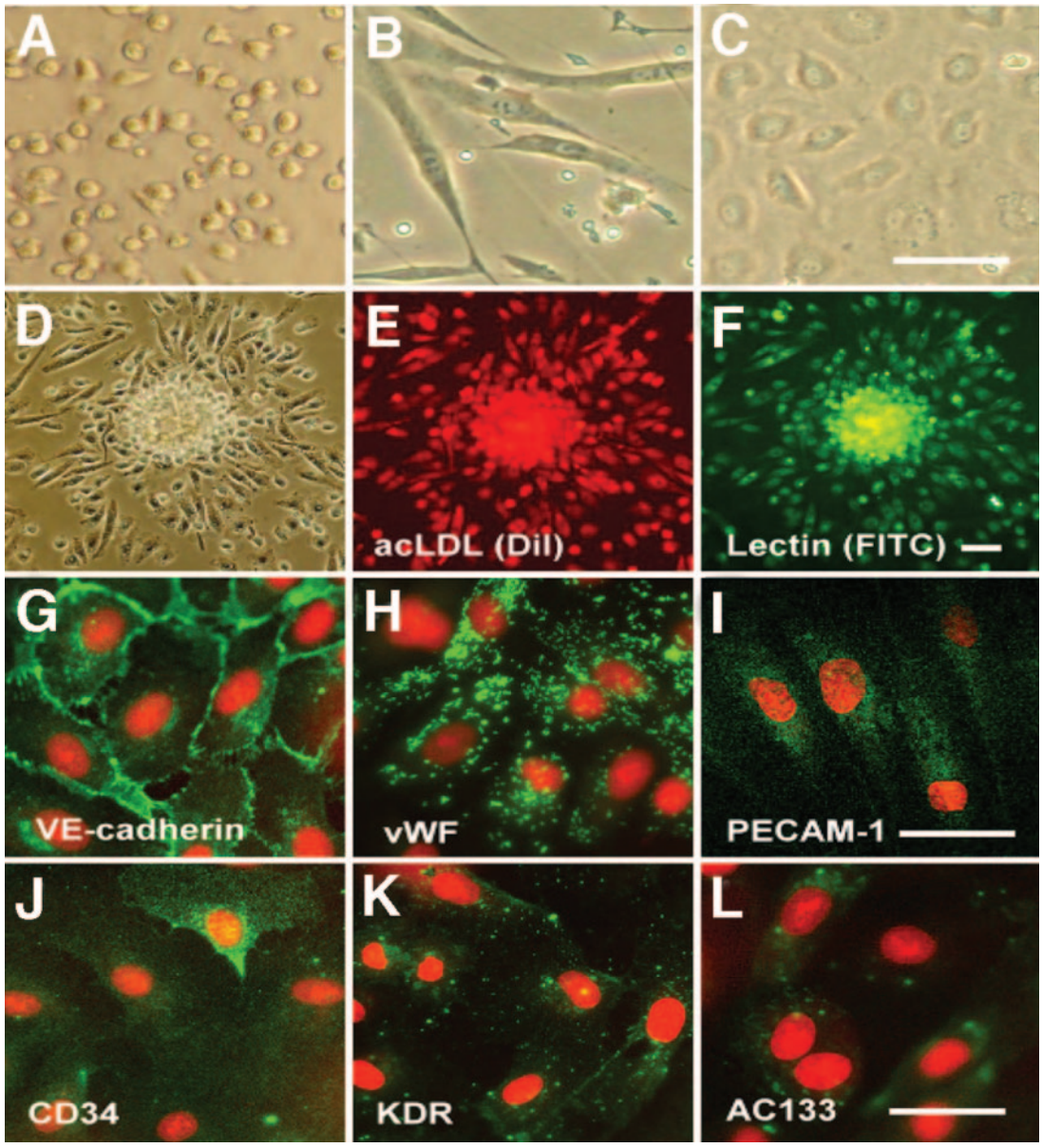

FIG. 1. Morphology and characterization of EPCs from peripheral blood. MNCs were isolated and plated on fibronectin-coated culture dish on the first day (*A*). Four days after plating, adherent early EPCs with spindle shape were shown (*B*). Twenty-one days after plating, late EPCs with cobblestone-like morphology were selected, reseeded, and grown to confluence (*C*). Phenotyping of endothelial characteristics of colony-forming units of EPCs (*D–F*). A colony of EPCs was defined as a central core of round cells with elongated sprouting cells at the periphery (*D*). Most cells were shown to simultaneously endocytose DiI-acLDL (red) (*E*) and bind fluorescein isothiocyanate UEA-1 (lectin) (green) (*F*). Immunofluorescence detection (green) of VE-cadherin (*G*), von Willebrand factor (*H*), PECAM-1 (platelet/endothelial cell adhesion molecule-1) (CD31) (*I*), CD34 (*J*), KDR (*K*), and AC133 (*L*) for late EPCs. Cells were counterstained with propidium iodide for nucleus (red). Scale bar: 50 μm.

cells with elongated sprouting cells at the periphery (Fig. 1*D*). EPC colony was further confirmed as cells double positive for acLDL uptake (Fig. 1*E*) and lectin (UEA-1) binding affinity (Fig. 1*F*). Late EPC characterization was performed by immunuhistochemical staining, the majority of the cells expressed mature endothelial markers VE-cadherin (Fig. 1*G*), von Willebrand factor (Fig. 1*H*), and PECAM-1 (platelet/endothelial cell adhesion molecule-1) (CD31) (Fig. 1*I*). In addition, CD34 (Fig. 1*J*), KDR (Fig. 1*K*), and AC133 (Fig. 1*L*) are considered critical markers of outgrowth endothelial cell–producing late EPCs, which are different from hematopoietic progenitors or leukocytes (4,5). Because AC133 rapidly disappeared, CD34/KDR double positive may be an important marker of EPCs in vitro (7).

High glucose decreases EPC number, proliferation, and colony-forming capacity. After seeding MNCs on wells, cells were incubated with different concentrations of glucose or mannitol (serving as osmotic control) for 4 days. Incubation of cells with glucose decreased the number of differentiated, adherent, early EPCs in a dose-dependent manner. As compared with that in control medium (5 mmol/l glucose), the amount of early EPCs assessed by fluorescein isothiocyanate lectin and DiI-acLDL staining was significantly reduced, by 26.4 and 33.6% in 20 and 25 mmol/l high-glucose medium, respectively ($P < 0.05$) (Fig. 2*A*). The effect of glucose on late EPC proliferation was analyzed by MTT assay. Glucose concentration dependently inhibited EPC proliferation activity, which became apparent at 20 mmol/l and maximal

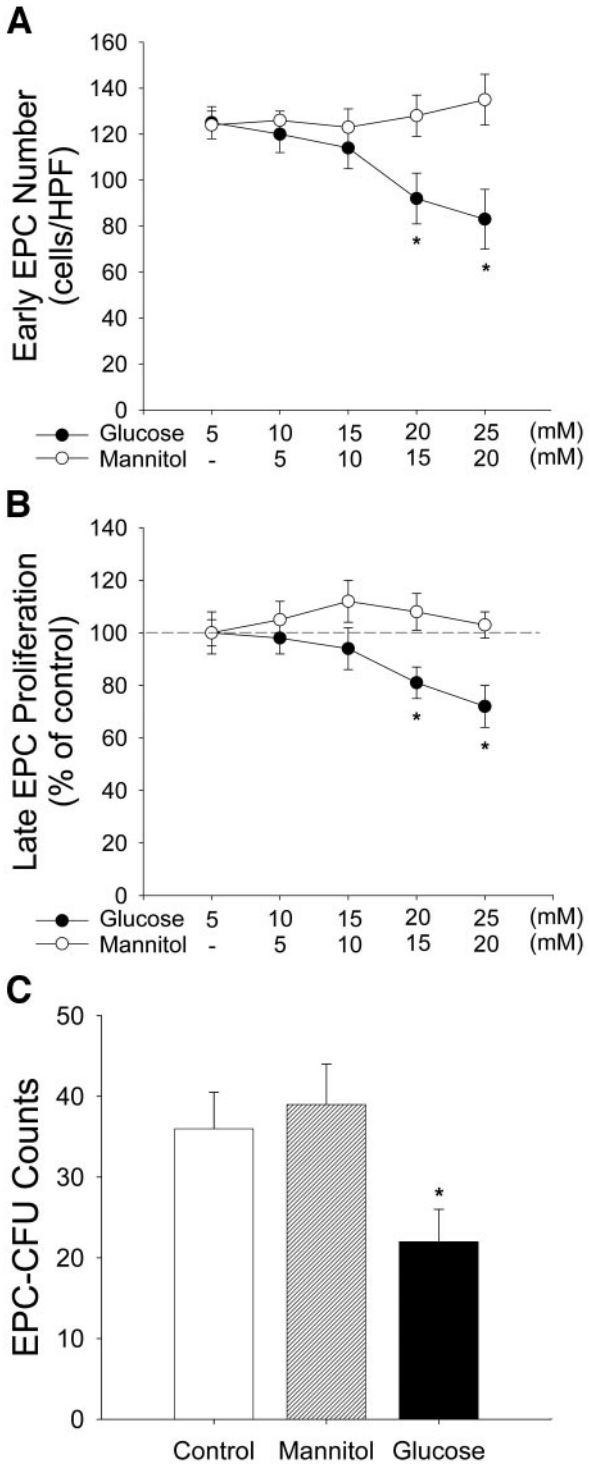

FIG. 2. High glucose decreases EPC number, proliferation, and colony-forming capacity. Cells were isolated and incubated with different concentrations of glucose or mannitol (osmotic control) for 4 days. Early EPCs were characterized as adherent cells that were dual positive for lactin staining and DiI-acLDL uptake. The numbers of EPCs were counted under microscope, and data are expressed as mean numbers of EPCs per high-power field (HPF) ± SE (*A*). MTT assay was also performed for late EPC proliferation activity (*B*), normalized to cells incubated in control medium (5 mmol/l glucose). Colony-forming assay for control, mannitol- (20 mmol/l), and glucose- (25 mmol/l) incubated EPCs (*C*). Data are expressed as means ± SE; $n = 5$, *$P < 0.05$ vs. control.

at 25 mmol/l (19.1 and 28.3% inhibition, respectively, $P < 0.05$) (Fig. 2*B*). The capacity of MNCs to form colonies capable of endothelial cell maturation and proliferation was stimulatory impaired by high-glucose incubation (25 mmol/l for 5 days, 38.6% inhibition, $P < 0.05$); by contrast, the osmotic control with mannitol did not change the colony-forming units of EPCs (P = NS) (Fig. 2*C*). The time-dependent effects of high glucose (25 mmol/l) on the number and cell proliferation of EPCs were also studied. Long-term (≥4 days) incubation with high glucose could significantly decrease the number of adherent early EPCs and inhibit the proliferation activity of late EPCs (data not shown).

High glucose increases senescence and impairs the migratory and tube formation function of EPCs. To determinate the onset of cellular aging, acidic β-galactosidase was detected as a biochemical marker for acidification typical of the EPC senescence (30). Compared with control medium (5 mmol/l glucose), incubation of either early or late EPCs with 25 mmol/l high glucose for 4 days significantly enhanced the percentage of senescence-associated β-galactosidase–positive EPCs, whereas an osmotic control with 20 mmol/l mannitol did not significantly influence the early/late EPC senescence (Fig. 3*A*). The data indicated that EPC senescence could be enhanced by high-glucose but not mannitol cultivation.

The migratory function of EPCs in response to VEGF is believed to be important during neovascularization (16), and late EPCs have been shown to exhibit better migratory capacity than early EPCs in vitro (7). The effect of high glucose on late EPC migration was evaluated using a modified Boyden chamber assay using VEGF as a chemoattractic factor. After 4 days of culturing, VEGF-induced augmentation of late EPC migration was significantly reduced, by 37.0%, in the high-glucose–treated group compared with the control group ($P < 0.05$), whereas mannitol did not influence the late EPC migration (P = NS) (Fig. 3*B*).

It has been shown that late EPCs but not early EPCs make capillary network formation on Matrigel successfully (6). An in vitro angiogenesis assay was performed with late EPCs to investigate the effect of glucose on EPC neovascularization. After 4 days of culturing, the functional capacity for tube formation of late EPCs on ECMatrix gel was significantly reduced in the high-glucose–treated group compared with the control group (62.2 ± 7.1 vs. 100 ± 6.8%, $P < 0.05$), whereas the osmotic control with mannitol did not influence the late EPC tube formation capacity (P = NS) (Fig. 3*C*). These data provided in vitro evidence that high-glucose cultivation impairs the migration and vasculogenesis abilities of late EPCs.

High glucose decreases the phosphorylation of eNOS and bioavailable NO in EPCs. It has been shown that hyperglycemia may inhibit eNOS activity by posttranslational modification at the Akt site in endothelial cells (33). We therefore investigated the effects of high glucose on the NO system in EPCs. After 4 days of incubation, the eNOS phosphorylation at $Ser^{1,177}$ shown by immunoblotting was significantly decreased both in early and late EPCs that had been cultured with high-glucose medium compared with that in control conditions (Fig. 4*A*). This reduction in eNOS phosphorylation was associated with a decrease in EPC-derived NO production (nitrite levels; 35.6 and 45.7% inhibition in early and late EPCs, respectively, $P < 0.05$) (Fig. 4*B*) and intracellular cGMP levels (38.1 and 40.8% inhibition in early and late EPCs, respec-

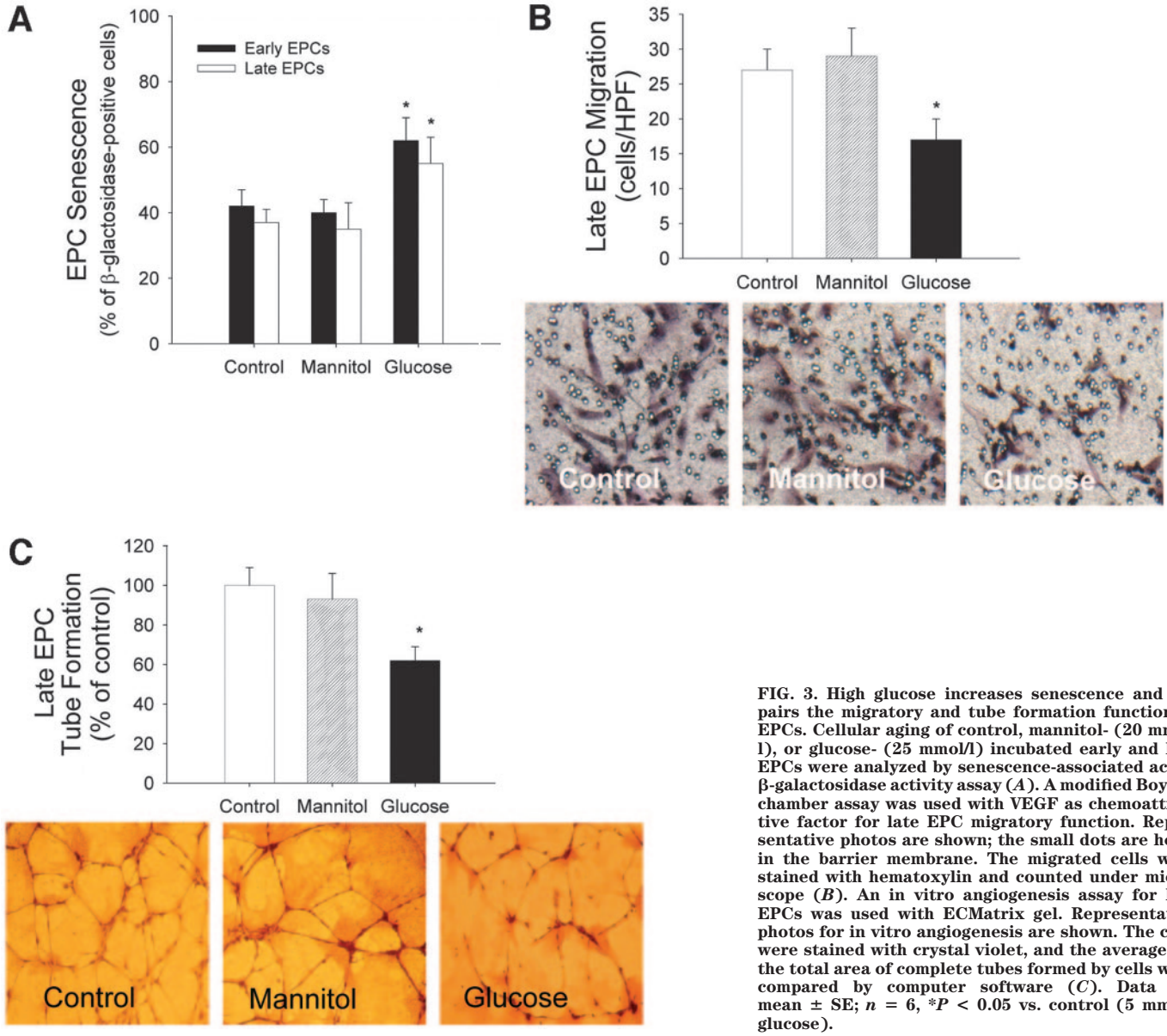

FIG. 3. High glucose increases senescence and impairs the migratory and tube formation function of EPCs. Cellular aging of control, mannitol- (20 mmol/l), or glucose- (25 mmol/l) incubated early and late EPCs were analyzed by senescence-associated acidic β-galactosidase activity assay (*A*). A modified Boyden chamber assay was used with VEGF as chemoattractive factor for late EPC migratory function. Representative photos are shown; the small dots are holes in the barrier membrane. The migrated cells were stained with hematoxylin and counted under microscope (*B*). An in vitro angiogenesis assay for late EPCs was used with ECMatrix gel. Representative photos for in vitro angiogenesis are shown. The cells were stained with crystal violet, and the averages of the total area of complete tubes formed by cells were compared by computer software (*C*). Data are mean ± SE; $n = 6$, *$P < 0.05$ vs. control (5 mmol/l glucose).

tively, $P < 0.05$) (Fig. 4*C*) without altering the total eNOS or inducible NOS (iNOS) expression, suggesting the selective inhibition of eNOS activity by high glucose. The osmotic control with mannitol did not influence either the phosphorylation or the expression of eNOS and iNOS in early or late EPCs.

Role of Akt/FoxO1, NO, p38 MAPK, and oxidative stress signalings in high-glucose–downregulated EPCs. We then investigated the role of Akt/FoxO1 activity in glucose toxicity effects on EPC differentiation (19). After 4-day incubation, the Akt and FoxO1 phosphorylation shown by immunoblotting was significantly decreased in both early and late EPCs cultured with high-glucose medium (Fig. 5*A*). The potential roles of PI3K/Akt-, NO-, and p38 MAPK–related mechanisms were also examined. Coincubation with NO donor sodium nitroprusside (SNP) or p38 MAPK inhibitor SB230580 significantly ameliorated the inhibitory effect of high glucose on EPC number and proliferation (for early and late EPCs, respectively). In contrast, coincubation with NOS inhibitor L-N^g-nitro-L-arginine methyl ester (L-NAME) or PI3K inhibitor LY294002 significantly enhanced the inhibitory effect of high glucose on EPC number/proliferation (Fig. 5*B*). These data indicated that high glucose may downregulate EPCs by modulating PI3K/Akt-, NO-, and p38 MAPK-related mechanisms.

In addition, the potential role of oxidative stress in high glucose–induced impairment of EPCs was investigated with the optimal concentrations of diverse antioxidants base on the literatures and our pilot studies. As shown in Fig. 5*C*, coincubation with various antioxidants, including membrane-permeable antioxidative enzymes (polyethylene glycol/PEG-SOD and PEG-catalase), vitamin C, glutathione precursor (*N*-acetylcysteine/NAC), pyrrolidine dithiocarbamate (PDTC), NADPH oxidase inhibitors (diphenyleneiodonium/DPI and apocynin), and mitochondrial complex I inhibitor (rotenone), failed to reverse the inhibitory effect of high glucose on EPC number/proliferation. Interestingly, PDTC and DPI (two antioxidants that also inhibit NO production), apocynin, and rotenone even dramatically inhibited the proliferation of EPCs. It was then suggested that high glucose downregulated EPCs by impairing NO- rather than by activating ROS-related mechanisms.

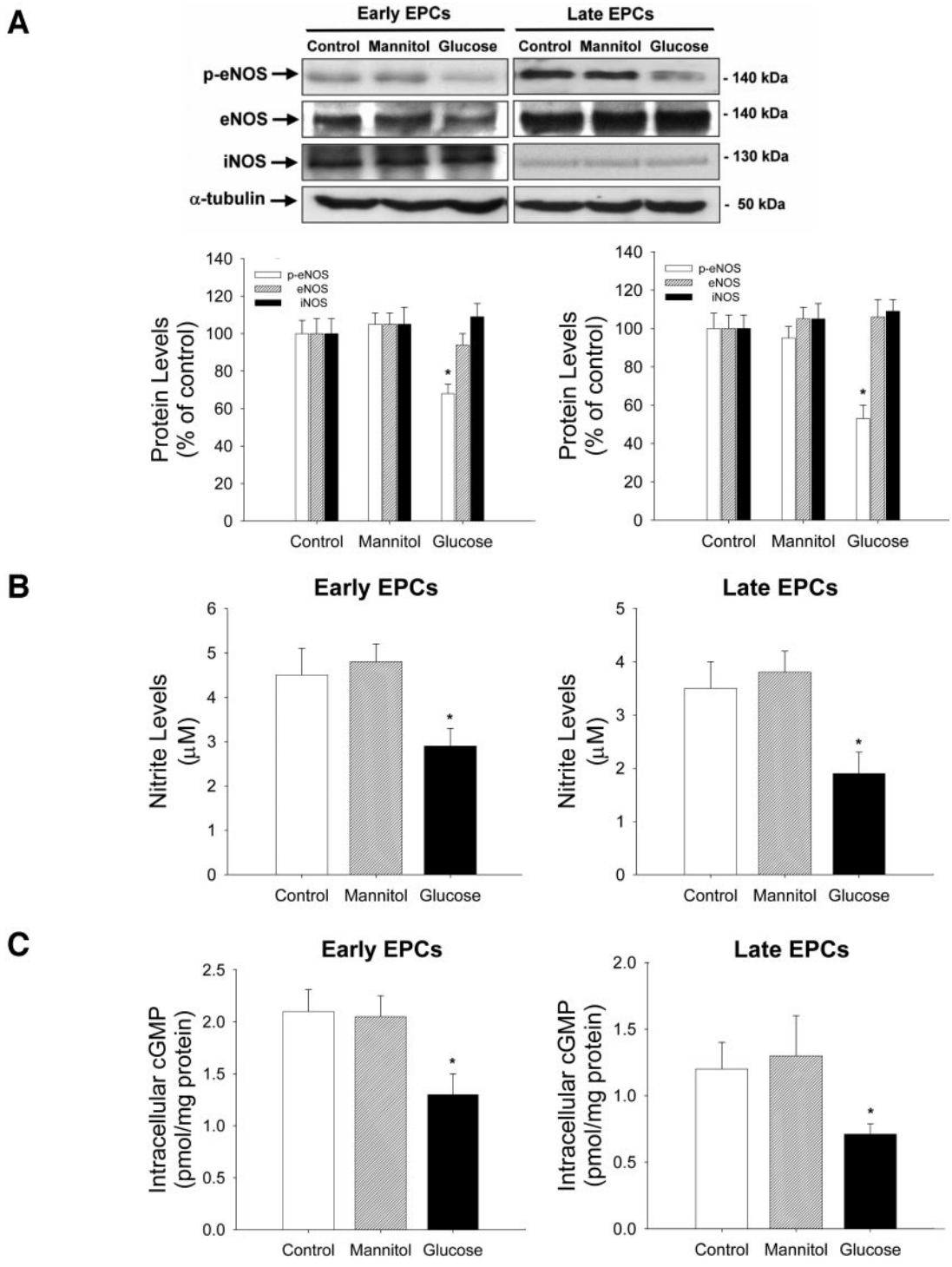

FIG. 4. High glucose decreases eNOS phosphorytion and bioavailable NO in EPCs. After incubation of EPCs with glucose or mannitol for 4 days, NOS protein levels, NO content, and cGMP formation were analyzed. A representative immunoblot shows the *p*-eNOS, eNOS, iNOS, and α-tubulin amounts of early and late EPCs in response to high glucose for 4 days. Each bar graph shows the summarized data from four separate experiments by densitometry after normalization to α-tubulin (*A*). Nitrite production (as NO content) in early/late EPC culture medium was measured by Griess reagent (*B*). Intracellular cGMP (pmol/mg protein) in early/late EPCs was measured by using enzyme-linked immunosorbent assay kits (*C*). Data are means ± SE; $n = 5$, *$P < 0.05$ vs. control (5 mmol/l glucose).

DISCUSSION

The major findings of this study included that *1*) long-term exposure to high glucose inhibited EPC colony-forming ability and reduced the number and proliferation activity but enhanced the senescence of early and late EPCs; *2*) long-term exposure to high glucose impaired the migration and vasculogenesis activities of late EPCs; *3*) in both early and late EPCs, Akt, FoxO1, and eNOS phosphorylation and bioavailable NO were significantly reduced in the long-term presence of high glucose; and *4*) the inhibitory effects of high glucose on EPC could be reversed by NO donor but not by various antioxidants. The proposed possible schema of this regulatory event is shown in Fig. 6. The novel findings suggested that impaired NO mecha-

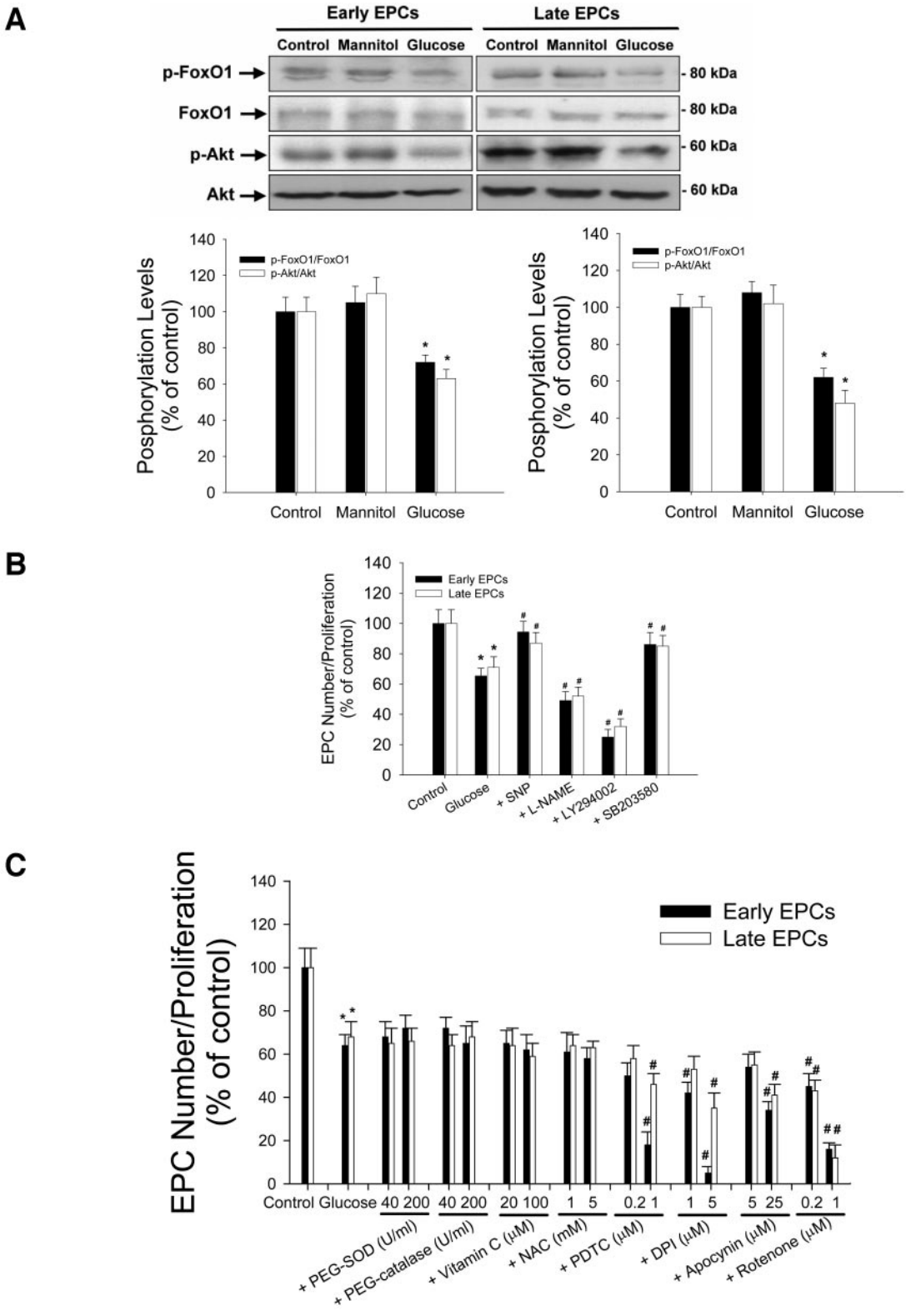

FIG. 5. Roles of Akt/FoxO1, NO, p38 MAPK, and oxidative stress signalings in high glucose–downregulated EPCs. A representative immunoblot shows the p-FoxO1/FoxO, and p-Akt/Akt amounts of early/late EPCs in response to high glucose for 4 days. Each bar graph shows the summarized data from four separate experiments by densitometry after normalization (*A*). EPC number and proliferation (for early and late EPCs, respectively) were anylyzed after for 4 days of culture in control or glucose/mannitol medium in the absence or presence of SNP (NO donor, 25 μmol/l), L-NAME (NOS inhibitor, 100 μmol/l), LY294002 (PI3K/Akt inhibitor, 5 μmol/l), and SB203580 (p38 MAPK inhibitor, 5 μmol/l) (*B*) or various concentrations of diverse antioxidants (PEG-SOD, PEG-catalase, vitamin C, NAC, PDTC, DPI, apocynin, and rotenone) (*D*). Data are means ± SE; $n = 5$, *$P < 0.05$ vs. control (5 mmol/l glucose), #$P < 0.05$ vs. high glucose–treated group.

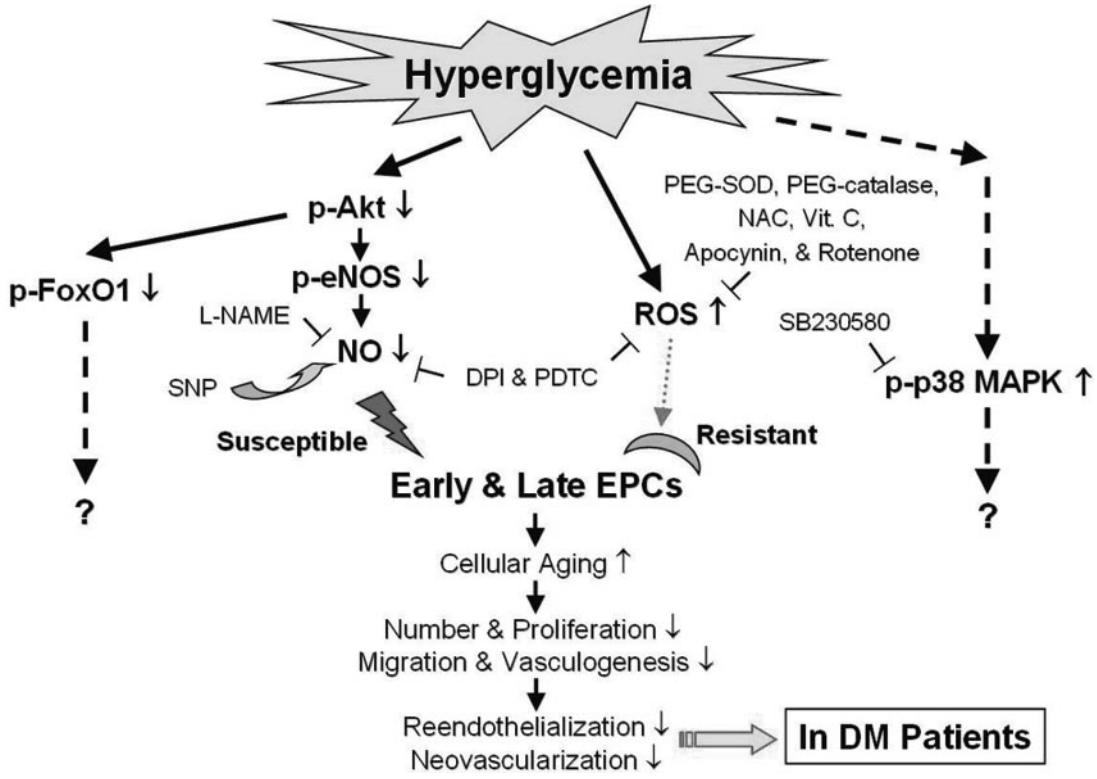

FIG. 6. Schematic representation of hyperglycemia-induced early and late EPC downregulation. High glucose impaired the proliferation and function of early and late EPCs. NO donor but not antioxidants reversed the impairments, suggesting the role of NO-related rather than ROS-mediated mechanisms in hyperglycemia-caused EPC downregulation. DM, diabetic.

nisms rather than increased oxidative stress could contribute to the reduction of number/proliferation and functional activity of both early and late EPCs in the long-term presence of high glucose.

It has been recognized that at least two different types of EPCs, early and late EPCs, could be classified in ex vivo culture system according to their time-dependent appearance (6,7). They share some endothelial phenotype but show different morphology, proliferation rate, survival features (6), and functions in neovascularization (7). Previous studies have shown that the number was reduced and the migration as well as vasculogenesis capacities were impaired in EPCs from patients with type 1 (12) and type 2 (10,11) diabetes. However, little data had been provided for the individual effect of high glucose on different types of EPCs. In this ex vivo study, we for the first time demonstrated the detrimental effect of high glucose on the senescence, number/proliferation, and the migration/vasculogenesis activities of both early and late EPCs, indicating the global harmful effect of high glucose on EPCs. The impairments could not be attributed to osmotic stress because the effects were not observed in mannitol-treated EPCs. Our findings on late EPCs may be of particular clinical interest because they could present the vasculogenesis capacity and may serve as a potential therapeutic target for vascular regeneration.

Stem and progenitor cells are considered as highly potent regenerating cells with high proliferation ability. However, their proliferation may be essentially dependent on aspects of the cellular environment such as cell-to-cell communication or growth factor support (30). It has been shown that in ex vivo cultivation, the onset of EPC senescence could be associated with a very low proliferation capacity and profoundly impaired clonal expansion potential (30). In the present study, long-term incubation with high glucose not only decreased the number and proliferation of early and late EPCs, but also accelerated the onset of cellular senescence (18), which appear to involve the NO-mediated pathways because NO donor SNP reversed and eNOS inhibitor L-NAME enhanced the inhibitory effect of high glucose on both early and late EPCs. Additionally, our findings were also compatible with recent suggestions that p38 MAPK may play a pivotal role in regulating the number of EPCs in the presence of high glucose or cytokines (17).

It has been suggested that high glucose may impair eNOS expression or its phosphorylation at the site $Ser^{1,177}$, resulting in reduced NO production, which is associated with reduced proliferation and an increase in apoptosis of endothelial cells and potentially contributes to the development of endothelial dysfunction and atherosclerosis in diabetes (33,34). In the present study, high glucose significantly reduced eNOS phosphorylation and bioavailable NO without affecting total eNOS and iNOS expression, suggesting that the posttranslational mechanisms are impaired by high glucose in both early and late EPCs. It is partially similar to what was demonstrated in mature vascular endothelial cells. Recently, EPCs were shown to express lower levels of eNOS as compared with mature vascular endothelial cells (28,29), and transplantation of autologous EPCs overexpressing eNOS in injured vessels enhanced the vasculoprotective properties of reconstituted endothelium (35). On the other hand, VEGF or erythropoietin could increase circulating progenitor cells by activating eNOS predominantly through Akt-dependent eNOS phosphorylation (15,33,36). Supplement of statins could also enhance endothelial NO production and reduce the senescence of EPCs (30). More interestingly, it was

shown that erythropoietin and benfotiamine could counteract glucose toxicity effects on EPC differentiation via Akt/FoxO1 signaling (19). All of the above indicate the critical role of NO mechanisms in maintaining EPC functions. Taking them together, one may speculate that the reduction of eNOS phosphorylation and NO production in the presence of high glucose could contribute to the impairments of EPCs and EPC-related vascular repair that might be observed in clinical diabetes (10,12).

It has been shown that ROS may play a key role in high glucose–induced apoptosis of mature vascular endothelial cells, which could be reversed by either eNOS activation or antioxidants (37,38). It was also suggested that hyperglycemia alone, through the mitochondrial (39–41) and/or NAD(P)H oxidase-mediated (42) overproduction of ROS, can induce changes in gene expression and the behavior of vascular endothelial cells in diabetes. Vitamin C and NAC, two water-soluble antioxidants, could enter mitochondria and confer mitochondrial protection against oxidative injury (43) and prevent hyperglycemia-induced apoptosis in human aortic endothelial cells (38). Strategies aiming at reducing hyperglycemia-induced ROS have been suggested as an useful adjuvant to antihyperglycemic therapies in the restoration of vasculogenesis and the prevention of diabetes complications (44). Though hyperglycemia-induced overproduction of ROS was used to explain EPC impairments observed in clinical diabetes (44), there is, however, no direct experimental evidence showing that the impairments of EPC function could be reversed by decreasing intracellular ROS. In the present study, the additional supplement with vitamin C (final concentration >100 μmol/l), NAC, or cell-permeable antioxidant enzymes all failed to ameliorate the adverse effects of high glucose on both early and late EPCs, suggesting the response of EPCs to antioxidants could be different from that of mature endothelial cells. In fact, it has been shown that the expression of antioxidant enzyme is enhanced and the tolerability to exogenous oxidative stress increased in EPCs (28,29), which may contribute to the survival of EPCs within the oxygen-poor environment of the bone marrow and to their ability to engraft within ischemic tissue during vasculogenesis (44). On the other hand, it was recently shown that endothelial cell apoptotic bodies may enhance the number and differentiation of EPCs (45) and hydrogen peroxide produced by angiopoitein-1 mediates angiogenesis (46), further suggesting that adequate oxidative stress might be required for the function of both early and late EPCs.

In the present study, chronic coincubation with PDTC (nuclear factor-κB inhibitor and antioxidant), DPI and apocynin (two agents with antioxidative property by inhibiting NADPH oxidase), or rotenone (mitochondrial complex I inhibitor) even significantly deteriorated high glucose–induced EPC impairment. Interestingly, PDTC and DPI are not only antioxidants but are also known as inhibitors of NOS mRNA translation (47) and flavoenzyme NOS (48), respectively. Compared with other antioxidants, they had more significant inhibitory effects on the number of early EPCs, suggesting that both intracellular NO- and oxidative stress–related mechanisms are particularly required for early EPCs in the long-term presence of high glucose. In fact, superoxide anion is a natural inhibitor of FAS-mediated cell death (49), and appropriate levels of ROS are essential and participate in important intracellular signaling (22). Our findings, different from the opinions of others (44), did support the concept that adequate ROS production may be helpful rather than harmful to EPC proliferation in the presence of high glucose. Accordingly, the main biochemical basis for high glucose–induced EPC dysfunction could be a complex other than excessive oxidative stress formation. This may provide a potential rationale for the dramatic failure of clinical trials with antioxidants for atherosclerosis in diabetic patients (50).

Similar with a previous study (17), a high-glucose (20–25 mmol/l) cell culture model was used to simulate clinical hyperglycemia for the in vitro evaluation of EPC function in the present study. The concentrations of glucose were 80–89 mg/dl (4.5–5 mmol/l) in the mediums as assayed by a clinical biochemical analyzer. The concentrations of glucose were 100 mg/dl (5–5.5 mmol/l) in diabetes (low glucose) and 434 mg/dl (20–24 mmol/l) in diabetes (high glucose). Thus, the high glucose concentration (20–25 mmol/l) used in the present study may correspond to 350–450 mg/dl of serum glucose levels, which may not present in well-controlled patients but might be seen after consuming a heavy meal in some poorly controlled diabetic patients. It could also happen in some stress conditions, such as those with inflammation or infection and during hospitalization. Thus, the findings of the present study may relevant for the worse clinical conditions that do obtain in some diabetic patients.

In conclusion, long-term presence of high glucose may enhance cellular senescence and decrease cell number/proliferation and functional competences in both early and late EPCs. Furthermore, impaired NO- rather than activated oxidative stress–related mechanisms could be the main contributor to high glucose–induced EPC dysfunction. These findings not only give further insights into the complex cellular mechanisms of EPCs for impaired vascular repair and abnormal neovasculogenesis in chronic hyperglycemia, but also provide a rationale for the potential therapeutic target for hyperglycemia-related vascular complications in diabetic patients.

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